PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF RECEIPT OF RECORD COPY

(PCT Rule 24.2(a))

To:

LEE, Han-Young
Seowon Building 1675-1
8th Floor
Seocho-dong
Seocho-gu
Seoul 137-070
RÉPUBLIQUE DE CORÉE

Date of mailing (day/month/year)	**IMPORTANT NOTIFICATION**
14 May 2001 (14.05.01)	
Applicant's or agent's file reference	**International application No.**
P0132-KAIST	PCT/KR01/00549

The applicant is hereby notified that the International Bureau has received the record copy of the international application as detailed below.

Name(s) of the applicant(s) and State(s) for which they are applicants:

KOREA ADVANCED INSTITUTE OF SCIENCE AND TECHNOLOGY (for all designated States except US)
LEE, Sang-Yup et al (for US)

International filing date	:	31 March 2001 (31.03.01)
Priority date(s) claimed	:	31 March 2000 (31.03.00)
Date of receipt of the record copy by the International Bureau	:	01 May 2001 (01.05.01)
List of designated Offices	:	

EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE,TR
National :CN,US

ATTENTION

The applicant should carefully check the data appearing in this Notification. In case of any discrepancy between these data and the indications in the international application, the applicant should immediately inform the International Bureau.

In addition, the applicant's attention is drawn to the information contained in the Annex, relating to:

- [X] time limits for entry into the national phase
- [X] confirmation of precautionary designations
- [ ] requirements regarding priority documents

A copy of this Notification is being sent to the receiving Office and to the International Searching Authority.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:

HA Ki-Nam

Telephone No. (41-22) 338.83.38

004020697

ANNEX TO FORM PCT/IB/301

International application No.
PCT/KR01/00549

INFORMATION ON TIME LIMITS FOR ENTERING THE NATIONAL PHASE

The applicant is reminded that the "national phase" must be entered before each of the designated Offices indicated in the Notification of Receipt of Record Copy (Form PCT/IB/301) by paying national fees and furnishing translations, as prescribed by the applicable national laws.

The time limit for performing these procedural acts is **20 MONTHS** from the priority date or, for those designated States which the applicant elects in a demand for international preliminary examination or in a later election, **30 MONTHS** from the priority date, provided that the election is made before the expiration of 19 months from the priority date. Some designated (or elected) Offices have fixed time limits which expire even later than 20 or 30 months from the priority date. In other Offices an extension of time or grace period, in some cases upon payment of an additional fee, is available.

In addition to these procedural acts, the applicant may also have to comply with other special requirements applicable in certain Offices. **It is the applicant's responsibility** to ensure that the necessary steps to enter the national phase are taken in a timely fashion. Most designated Offices do not issue reminders to applicants in connection with the entry into the national phase.

For detailed information about the procedural acts to be performed to enter the national phase before each designated Office, the applicable time limits and possible extensions of time or grace periods, and any other requirements, see the relevant Chapters of Volume II of the PCT Applicant's Guide. Information about the requirements for filing a demand for international preliminary examination is set out in Chapter IX of Volume I of the PCT Applicant's Guide.

GR and ES became bound by PCT Chapter II on 7 September 1996 and 6 September 1997, respectively, and may, therefore, be elected in a demand or a later election filed on or after 7 September 1996 and 6 September 1997, respectively, regardless of the filing date of the international application. (See second paragraph above.)

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

CONFIRMATION OF PRECAUTIONARY DESIGNATIONS

This notification lists only specific designations made under Rule 4.9(a) in the request. It is important to check that these designations are correct. Errors in designations can be corrected where precautionary designations have been made under Rule 4.9(b). The applicant is hereby reminded that any precautionary designations may be confirmed according to Rule 4.9(c) before the expiration of 15 months from the priority date. If it is not confirmed, it will automatically be regarded as withdrawn by the applicant. There will be no reminder and no invitation. Confirmation of a designation consists of the filing of a notice specifying the designated State concerned (with an indication of the kind of protection or treatment desired) and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.

REQUIREMENTS REGARDING PRIORITY DOCUMENTS

For applicants who have not yet complied with the requirements regarding priority documents, the following is recalled.

Where the priority of an earlier national, regional or international application is claimed, the applicant must submit a copy of the said earlier application, certified by the authority with which it was filed ("the priority document") to the receiving Office (which will transmit it to the International Bureau) or directly to the International Bureau, before the expiration of 16 months from the priority date, provided that any such priority document may still be submitted to the International Bureau before that date of international publication of the international application, in which case that document will be considered to have been received by the International Bureau on the last day of the 16-month time limit (Rule 17.1(a)).

Where the priority document is issued by the receiving Office, the applicant may, instead of submitting the priority document, request the receiving Office to prepare and transmit the priority document to the International Bureau. Such request must be made before the expiration of the 16-month time limit and may be subjected by the receiving Office to the payment of a fee (Rule 17.1(b)).

If the priority document concerned is not submitted to the International Bureau or if the request to the receiving Office to prepare and transmit the priority document has not been made (and the corresponding fee, if any, paid) within the applicable time limit indicated under the preceding paragraphs, any designated State may disregard the priority claim, provided that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity to furnish the priority document within a time limit which is reasonable under the circumstances.

Where several priorities are claimed, the priority date to be considered for the purposes of computing the 16-month time limit is the filing date of the earliest application whose priority is claimed.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

LEE, Han-Young
Seowon Building 1675-1
8th Floor
Seocho-dong
Seocho-gu
Seoul 137-070
RÉPUBLIQUE DE CORÉE

Date of mailing (day/month/year) 14 May 2001 (14.05.01)	
Applicant's or agent's file reference P0132-KAIST	**IMPORTANT NOTIFICATION**
International application No. PCT/KR01/00549	International filing date (day/month/year) 31 March 2001 (31.03.01)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 31 March 2000 (31.03.00)

Applicant

KOREA ADVANCED INSTITUTE OF SCIENCE AND TECHNOLOGY et al

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3 An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
31 Marc 2000 (31.03.00)	2000/17052	KR	01 May 2001 (01.05.01)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

HA Ki-Nam

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

To:
LEE, Han-Young
Seowon Building 1675-1
8th Floor
Seocho-dong
Seocho-gu
Seoul 137-070
RÉPUBLIQUE DE CORÉE

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

Date of mailing (day/month/year) 04 October 2001 (04.10.01)	
Applicant's or agent's file reference P0132-KAIST	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/KR01/00549	31 March 2001 (31.03.01)	31 March 2000 (31.03.00)

Applicant
KOREA ADVANCED INSTITUTE OF SCIENCE AND TECHNOLOGY et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   CN,EP

   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   04 October 2001 (04.10.01) under No. WO 01/73081

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38

Continuation of Form PCT/IB/308

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

Date of mailing (day/month/year) 04 October 2001 (04.10.01)	IMPORTANT NOTICE
Applicant's or agent's file reference P0132-KAIST	**International application No.** PCT/KR01/00549

The applicant is hereby notified that, at the time of establishment of this Notice, the time limit under Rule 46.1 for making amendments under Article 19 has not yet expired and the International Bureau had received neither such amendments nor a declaration that the applicant does not wish to make amendments.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR01/00549

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)

IPC7 C12N 15/70

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

Electronic data base consulted during the international search (name of data base and, where practicable, search trerms used)

NCBI pubmed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Leukemia. 1993 Feb;7(2):310-7	1-4, 5-6, 7-10
A	Cytotechnology. 1991 Sep;7(1):25-32.	1-4, 5-6, 7-10
A	J Immunol. 1992 Jul 1;149(1):113-9.	1-4, 5-6, 7-10
A	Int J Hematol. 1995 Feb;61(2):61-8.	1-4, 5-6, 7-10

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevence

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 AUGUST 2001 (27.08.2001)	27 AUGUST 2001 (27.08.2001)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
Government Complex-Daejeon, Dunsan-dong, Seo-gu, Daejeon Metropolitan City 302-701, Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

LIM, Hea Joon

Telephone No. 82-42-481-5590

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR01/00549

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)

IPC7 C12N 15/70

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

Electronic data base consulted during the international search (name of data base and, where practicable, search trerms used)

NCBI pubmed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Leukemia. 1993 Feb;7(2):310-7	1-4, 5-6, 7-10
A	Cytotechnology. 1991 Sep;7(1):25-32.	1-4, 5-6, 7-10
A	J Immunol. 1992 Jul 1;149(1):113-9.	1-4, 5-6, 7-10
A	Int J Hematol. 1995 Feb;61(2):61-8.	1-4, 5-6, 7-10

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevence
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 AUGUST 2001 (27.08.2001)	27 AUGUST 2001 (27.08.2001)

Name and mailing address of the ISA/KR	Authorized officer
Korean Intellectual Property Office Government Complex-Daejeon, Dunsan-dong, Seo-gu, Daejeon Metropolitan City 302-701, Republic of Korea Facsimile No. 82-42-472-7140	LIM, Hea Joon Telephone No. 82-42-481-5590

Form PCT/ISA/210 (second sheet) (July 1998)

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in description
On page 7 , lines 18-25 .

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on additional sheet ☐

Name of depositary institution

Korean Collection for Type Cultures (KCTC)

Address of depositary institution (including postal code and country)

Korea Research Institute of Bioscience and Biotechnology(KRIBB)
#52, Oun-dong, Yusong-ku,
Taejon 305-333,
Republic of Korea

Date of deposit	Accession Number
Mar. 13, 2000	KCTC 0754BP

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information continues on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1998)

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : LEE, Sang Yup
Expo Apt. 212-702, #464-1, Chunmin-dong, Yusong-ku, Taejon 305-390,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli **MC4100/pTHKCSFmII**	**KCTC 0754BP**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
(Mark with a cross where applicable)

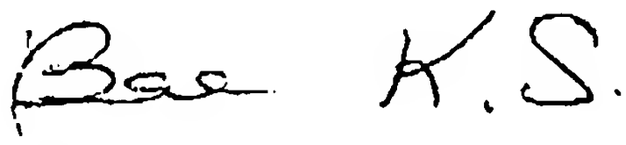
III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **March 13 2000.**

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: **Korean Collection for Type Cultures**	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):
Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	 BAE, Kyung Sook, Director Date: **March 16 2000**

(12) INTERNATIONAL A ATION PUBLISHED UNDER THE PATEN OPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 October 2001 (04.10.2001)

PCT

(10) International Publication Number
WO 01/73081 A1

(51) International Patent Classification[7]: C12N 15/70

(21) International Application Number: PCT/KR01/00549

(22) International Filing Date: 31 March 2001 (31.03.2001)

(25) Filing Language: Korean

(26) Publication Language: English

(30) Priority Data:
2000/17052 31 March 2000 (31.03.2000) KR

(71) Applicant *(for all designated States except US)*: **KOREA ADVANCED INSTITUTE OF SCIENCE AND TECHNOLOGY** [KR/KR]; 373-1, Kusong-dong, Yusong-gu, Taejon 305-701 (KR).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **LEE, Sang-Yup** [KR/KR]; 212-702 Expo Apartment, Chonmin-dong, Yusong-gu, Taejon 305-390 (KR). **JEONG, Ki-Jun** [KR/KR]; 102-411 Kaist Apartment, Kung-dong, Yusong-gu, Taejon 305-335 (KR).

(74) Agent: **LEE, Han-Young**; Seowon Building 1675-1, 8th Floor, Seocho-dong, Seocho-gu, Seoul 137-070 (KR).

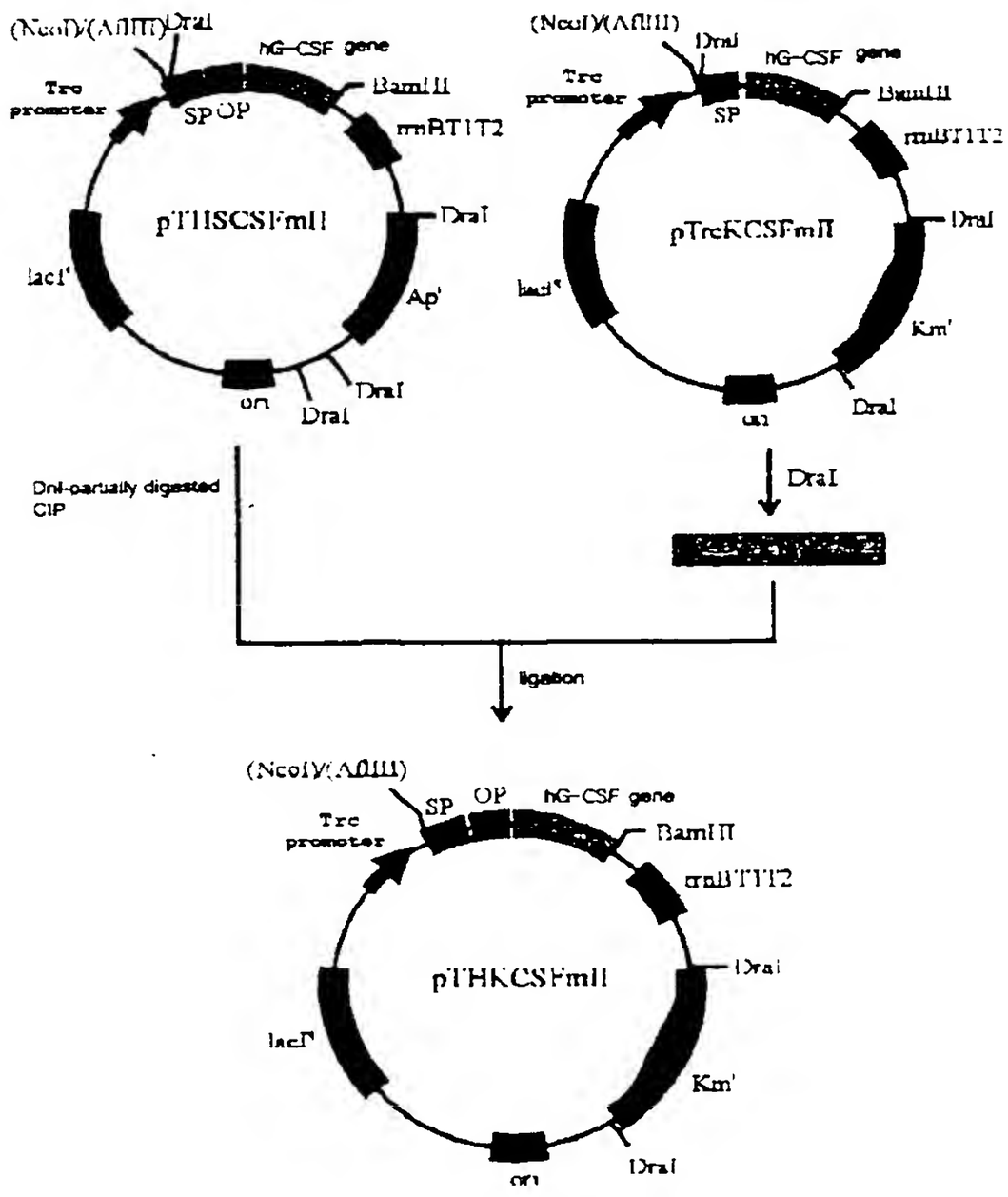
(81) Designated States *(national)*: CN, US.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

[Continued on next page]

(54) Title: ESCHERICHIA COLI STRAIN SECRETING HUMAN GRANULOCYTE COLONY STIMULATING FACTOR (G-CSF)



(57) Abstract: The present invention provides a recombinant plasmid vector comprising a kanamycin resistance gene, a promoter, an endoxylanase signal sequence, a nucleotide sequence coding for an oligopeptide consisting of 13 amino acids including 6 consecutive histidine residues, and a human granulocyte colony stimulating factor (hG-CSF) gene; an *E. coli* transformed with the said vector; and, a process for producing complete hG-CSF protein with high purity from the protein pool secreted by the said microorganism. In accordance with the invention, the hG-CSF protein can be prepared with high purity through rather simple process facilitating secretion of large amount of hG-CSF fusion protein into the periplasm, which does not require complicated processes such as solubilization and subsequent refolding required for isolation of the hG-CSF protein produced in cytoplasm as insoluble inclusion bodies by conventional techniques, thus, the hG-CSF protein can be widely used as an active ingredient in the development of supplementary agents for anticancer therapy.

WO 01/73081 A1

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

ESCHERICHIA COLI STRAIN SECRETING HUMAN GRANULOCYTE COLONY STIMULATING FACTOR(G-CSF)

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to an *E. coli* producing and secreting human granulocyte-colony stimulating factor(hG-CSF), more specifically, to a recombinant plasmid constructed to express secretory hG-CSF in *E. coli*, an *E. coli* transformed with the said plasmid to secrete hG-CSF, and a process for preparing hG-CSF using the said transformed *E. coli*.

Description of the Prior Art

Colony stimulating factors(CSFs) known to be synthesized by various cell types such as mononuclear macrophages, T-lymphocytes, and fibroblasts are found in the various parts of normal human body. CSFs are classified into three major categories, granulocyte-colony stimulating factor(G-CSF), macrophage-colony stimulating factor(M-CSF) and granulocyte/macrophage-colony stimulating factor(GM-CSF), among them, G-CSF is an essential protein in manufacturing blood cells via promoting proliferation and differentiation of hemopoietic stem cells, and facilitates increase in numbers of granulocytes, especially, neutrophils which play an important role in the protection of the body from the infection. Chemotherapies widely used to treat growing tumors not only inhibit the growth of tumors but also inhibit the production of neutrophils, giving rise to severe side effects due to the diminished protecting function of neutrophils. Administration of G-CSF to the patients under such chemotherapies is known to be an effective way of treatment and prevention of the

infectious diseases by means of facilitating the increase in neurophil numbers.

In 1986, the hG-CSF gene was isolated from a human squamous carcinoma cell line CHU-II, its nucleotide sequence was first determined and expressed in COS cells by Nagata et al.(see: Nagata et al., *Nature* 319: 415 (1986)). The hG-CSF is a glycoprotein comprising 30 signal peptides which consists of 174 amino acid residues. The hG-CSF includes 5 cysteine residues of which 4 cysteines form two disulfide bonds, between Cys-36 and Cys-64, and between Cys-64 and Cys-74, which serve for folding of the expressed protein and its activity(see: Hill et al., *Proc. Natl. Acad. Sci.*, USA, 90:5167-5171(1993)). The hG-CSF dose not have the consensus sequence(Asn-X-Thr/Ser) for N-glycosylation, but O-glycosylation occurs at Thr-133. However, the recombinant G-CSF produced in *E. coli* is known to have almost the same biological activities as natural G-CSF, which means glycosylation is unnecessary for the G-CSF activity.

With the recent progress in recombinant DNA technology, G-CSF can be produced in bacteria, plant cells and animal cells, and some results previously reported are described here: Souza et al. isolated a cDNA from the human bladder cancer cell line 5637, determined its sequence and reported its expression in *E. coli*(see: Souza et al., *Science*, 232:61(1986)). Moreover, researches on production of hG-CSF in *E. coli*, plant cells and animal cells, and on construction and production of hG-CSF derivatives have been reported. However, technologies known to date for the production of hG-CSF in *E. coli* have many disadvantages in terms of protein yield or production cost since the hG-CSF is produced in cytoplasm in the form of insoluble inclusion body, which requires subsequent solubilization and renaturation to obtain biologically active form of hG-CSF protein. Although small quantity of soluble hG-CSF can be isolated directly, such method still have limitations in a sense that the active fraction of hG-CSF protein has to be

isolated from the pool of enormous amounts of *E. coli* proteins.

In general, proteins secreted to the periplasm of *E. coli* carry signal sequence, which is found in all proteins transportable out of the cytoplasm, and cleaved off by signal peptidase in the periplasm. The signal sequence is essential in secreting proteins in *E. coli*. Therefore, recombinant proteins originally not encoded by *E. coli* genes can be secreted into the periplasm or to the extracellular broth by joining known signal sequence(OmpA, OmpF, PelB, PhoA, SpA, etc.), as it is or with slight modifications, to the N-terminus of gene coding an exogenous protein.

The method for production of hG-CSF by secretion into the periplasmic space has following advantages over the conventional method by producing in cytoplasm described above: first, it is easy to isolate and purify the recombinant proteins, with high purity, in periplasm than in cytoplasm, since there are fewer proteins in periplasm than in cytoplasm(see: Nossal, N.G. et al., *J. Biol. Chem.*, 241:3055-3062(1966)); secondly, recombinant proteins secreted into periplasm are segregated from the cytoplasm where the most proteases are found, obtaining high yield in production of recombinant protein by avoiding degradation of the protein by proteases present in cytoplasm(see: Meerman and Georgiou, *Ann. N.Y. Acad. Sci.*, 721:292-302(1994)); thirdly, the bacterial periplasm is more oxidizing environment than cytoplasm, conducting disulfide bond formation and correct folding of polypeptide easily, thus, the formation of insoluble aggregates is avoidable(see: Hockney, *TIBTECH*, 12:456-463(1994)).

Having such advantages, the method resulting in secretion of recombinant proteins into the periplasm has been employed for production of hG-CSF in *E. coli* and reported as follows: Perez-Perez et al. have tried to get secreted form of hG-CSF, employing OmpA which is one of the signal sequences in *E. coli*, without success. To solve that

problem, they employed the system for coexpression of two molecular chaperones, DnaK and DnaJ, and merely obtain a small quantity of secreted hG-CSF(see: Perez-Perez et al., *Biochem. Biophys. Res. Commun.*, 210:524-529(1995));and, Chung et al. have tried to obtain secreted form of hG-CSF employing another signal sequence, PelB, again without success, but hG-CSF was accumulated in the form of insoluble inclusion body in cytoplasm(see: Chung et al., *J. Fermen. Bioengin.*, 85:443-446(1998)).

In view of above situation, there is a continuing need to develop the technique for facilitating secretion of hG-CSF at a substantial level into the periplasm through rather simple process which does not require solubilization of insoluble inclusion body and subsequent refolding.

SUMMARY OF THE INVENTION

The present inventors have made an effort to develop the technique for facilitating secretion of hG-CSF into the periplasm, thus, they have found that *E. coli*, transformed with the recombinant plasmid containing nucleotide sequence coding for oligopeptide consisting of 13 amino acids, *Bascillus*-derived endoxylanase signal sequence and 6 histidine residues, can secrete oligopeptide/hG-CSF fusion protein efficiently so that hG-CSF protein can be produced using the said transformed *E. coli*.

The first object of the present invention is, therefore, to provide the recombinant plasmid constructed for *E. coli* to express and secrete hG-CSF fusion protein therefrom.

The second object of the invention is to provide transformed *E. coli* with the said plasmid.

The third of the invention is to provide a process for producing hG-CSF protein employing the said

microorganism.

BRIEF DESCRIPTION OF THE DRAWINGS

The above, the other objects and features of the invention will become apparent from the following descriptions given in conjunction with the accompanying drawings, in which:

Figure 1 is the nucleotide sequence of hG-CSF gene inserted into the plasmid p19CSF.

Figure 2 shows a construction scheme and a genetic map for the plasmid p19CSFm.

Figure 3 is the nucleotide sequence and the deduced amino acid sequence of hG-CSF gene inserted into the plasmid p19CSFm.

Figure 4 shows a construction scheme and a genetic map for the plasmid pEDCSFm.

Figure 5 is the nucleotide sequence and the deduced amino acid sequence of hG-CSF gene inserted into the plasmid pEDCSFm.

Figure 6 shows a construction scheme and a genetic map for the plasmid pEDCSFmII.

Figure 7 is the nucleotide sequence and the deduced amino acid sequence of the N-terminal portion of hG-CSF gene inserted into the plasmid pEDCSFmII.

Figure 8 shows a construction scheme and a genetic map for the plasmid pTrcSCSFmII.

Figure 9 is the nucleotide sequence and the deduced amino acid sequence of the N-terminal portion of hG-CSF gene inserted into the plasmid pTrcSCSFmII.

Figure 10 shows a construction scheme and a genetic map for the plasmid pTrcKCSFmII.

Figure 11 shows a construction scheme and a genetic map for the plasmid pTHSCSFmII.

Figure 12 is the nucleotide sequence and the deduced amino acid sequence of the N-terminal portion of hG-CSF gene inserted into the plasmid pTHSCSFmII.

Figure 13 shows a construction scheme and a genetic map for the plasmid pTHKCSFmII.

DETAILED DESCRIPTION OF THE INVENTION

The recombinant plasmid of the invention designed for secreting hG-CSF fusion protein efficiently from *E. coli* contains: a kanamycin resistance gene, an endoxylanase signal sequence, a nucleotide sequence coding for an oligopeptide consisting of 13 amino acid residues including 6 consecutive histidine residues, a modified hG-CSF gene and Trc promoter. The recombinant *E. coli* of the invention secreting hG-CSF protein efficiently is prepared by transformation of *E. coli* with the said recombinant plasmid. The hG-CSF protein can be prepared with high purity by isolating the hG-CSF fusion protein from the protein pool obtained from the transformed *E. coli* using a Ni-column and subsequent protease treatment.

The present invention is illustrated in more detail as followings.

First, the recombinant plasmid vector pTHKCSFmII, containing cDNA encoding hG-CSF protein, endoxylanase signal sequence for hG-CSF protein secretion, Trc promoter which is a strong inducible promoter, kanamycin resistance gene encoding non-secretory protein, and a nucleotide sequence encoding an oligopeptide consisting of 13 amino acid residues including 6 consecutive histidine residues between endoxylanase signal sequence and hG-CSF polypeptide, is constructed for preparing transformed *E. coli* which expresses and secretes hG-CSF fusion protein into the periplasm: wherein, cDNA encoding hG-CSF protein is

obtained by joining 3rd exon-deleted hG-CSF cDNA obtained from a human breast carcinoma cDNA library to the synthesized 3rd exon, *Bacillus* sp.-derived endoxylanase signal sequence is employed for facilitating secretion of hG-CSF protein from *E. coli*, and kanamycin resistance gene is employed as a selection marker. Furthermore, to prevent cell lysis occurred during the secretion of hG-CSF protein in *E. coli*, a nucleotide sequence encoding an oligopeptide consisting of 13 amino acid residues including 6 consecutive histidine residues was included between DNA of endoxylanase signal sequence and hG-CSF gene, and the amino acid sequence of the oligopeptide is N'-Ala-Gly-Pro-His-His-His-His-His-Ile-Glu-Gly-Arg-C'(SEQ ID NO: 1).

Subsequently, recombinant *E. coli* secreting hG-CSF fusion protein is prepared by introducing the plasmid pTHKCSFmII constructed above into *E. coli*: wherein, *E. coli* strains BL21(DE3), HB101, MC4100, W3110 and XL1-Blue, preferably MC4100, can be used. *E. coli* MC4100 transformed with the recombinant plasmid pTHKCSFmII was named *E. coli* MC4100/pTHKCSFmII(*Escherichia coli* MC4100/pTHKCSFmII), which was deposited with the Korean Collection for Type Cultures(KCTC) affiliated to Korea Research Institute of Bioscience and Biotechnology(KRIBB), an international depository authority, under accession(deposition) No. KCTC 0754BP on Mar. 13, 2000.

Finally, the complete hG-CSF protein is prepared from the protein pool obtained from the transformed *E. coli* using a Ni-column and a protease: the said *E. coli* secretes hG-CSF fusion protein of which N-terminus is linked by an oligopeptide consisting of 13 amino acid residues including 6 consecutive histidine residues. The secreted hG-CSF fusion protein is isolated using a Ni-column to which 6 consecutive histidine residues present in the oligopeptide of the fusion protein can bind, and then the complete hG-CSF protein can be prepared from the hG-CSF fusion protein isolated above by treating a protease to get rid of the oligopeptide. Since the hG-CSF protein has to be non-

susceptible to the protease employed, the C-terminal sequence of the oligopeptide should be selected to be cleaved off by the protease of which recognition sequences are not present in the hG-CSF protein. As an example, in the present invention, the C-terminal amino acid sequence of the oligopeptide was selected to be Ile-Glu-Gly-Arg(see: SEQ ID NO: 1), which is recognized and cleaved by Factor Xa, a protease not having recognition sequences in hG-CSF protein.

The present invention is further illustrated in the following examples, which should not be taken to limit the scope of the invention.

Example 1: Preparation of cDNA for human granulocyte-colony stimulating factor(hG-CSF)

To isolate hG-CSF gene, hG-CSF cDNA was prepared by PCR amplification of a human breast carcinoma cDNA library. Nucleotide sequence of the hG-CSF gene was retrieved in the GenBank, and then, primer 1: 5'-GCGAATTCATGGCTGGACCTGCCACCCAG-3'(SEQ ID NO: 2) and primer 2: 5'-GCGGATCCTTATTAGGGCTGGGCAAGGTGGCG-3'(SEQ ID NO: 3) were synthesized, respectively. For the convenience of cloning of PCR product, *Eco*RI and *Bam*HI restriction sites were introduced at primer 1 and primer 2, respectively. Then, PCR was performed by, employing High Fidelity PCR System(Boehringer Mannheim Co., Germany), under following condition: one cycle of denaturation at 94℃ for 7 min; 30 cycles of denaturation at 94℃ for 1 min, annealing at 50℃ for 1 min, and extension at 72℃ for 1 min; and, one cycle of extension at 72℃ for 7 min. DNA molecules obtained by the PCR were subjected to agarose gel electrophoresis to isolate DNA fragment of approximately 530bp, which was subsequently digested with *Eco*RI and *Bam*HI to obtain DNA fragment concerned.

In order to clone the DNA fragment obtained above,

the said DNA fragment was joined into the plasmid pUC19(see: Yanisch-Perron et al., Gene, 33:109-119(1985)) digested with *Eco*RI and *Bam*HI using T4 DNA ligase, and then, the ligation product was introduced into *E. coli* XL1-Blue(*supE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lacF'*(*proAB+ lacIqlacZΔ M15Tn*(*tetr*)))(see: Bullock, W. O. et al., *BioTechniques*, 5:376-378(1987)) by electroporation technique to obtain transformed *E. coli*. The transformants were selected on the LB agar medium containing ampicillin(50 $\mu g/\ell$) and the recombinant plasmid p19CSF was obtained therefrom. The nucleotide sequence of the fragment in p19CSF was determined using automatic DNA sequencer(ABI Prism model 377, Perkin Elmer Co., U.S.A.). It has been found that there was 108bp deletion in the middle of the cloned fragment of the invention compare to the published nucleotide sequence of hG-CSF gene, meanwhile, the rest sequence of 508bp was identical to the published sequence of hG-CSF gene(see: Figure 1). Comparing with the sequence of genomic DNA of hG-CSF, the 108bp deletion in the fragment of the invention corresponded to the third exon among five exons of hG-CSF gene.

In order to obtain complete sequence of the hG-CSF gene, four primers, primer 3: 5'-TCCTCGGGGTGGCACAGCTTGTAGGTGGCACACAGCTTCTCCTGGAGCGC-3'(SEQ ID NO: 4), primer 4: 5'-GCTGTGCCACCCCGAGGAGCTGGTGCTGCTCGGACACTCTCTGGGCATCC-3'(SEQ ID NO: 5), primer 5: 5'-TGGCTGGGGCAGCTGCTCAGGGGAGCCCAGGGGATGCCCAGAGAGTGTC-3'(SEQ ID NO: 6), and primer 6: 5'-AGCAGCTGCCCCAGCCAGGCCCTGCAGCTGGCAGGCTGCTTGAGCCAA-3'(SEQ ID NO: 7) corresponding the third exon were prepared and PCR was carried out. Using primer 1 and the PCR product obtained above, p19CSF was subjected to PCR amplification, and with the primer 2, PCR was carried out in the same way as with primer 1. Using both PCR products, PCR was performed once again and the resulting product was digested with two restriction enzymes, *Eco*RI and *Bam*HI, and then the

fragments were cloned into the same site of pUC19 as the site used above. The transformed E. coli XL1-Blue were selected on the LB agar medium containing ampicillin(50 μg/ℓ) and the recombinant plasmid p19CSFm was obtained therefrom(see: Figure 2). The nucleotide sequence of the fragment in p19CSFm was determined and found to be identical to the sequence of the published hG-CSF gene(see: Figure 3).

Example 2: Construction of the recombinant plasmid, pEDCSFm

In order to get hG-CSF protein expressed from the hG-CSF gene obtained in Example 1, the mature hG-CSF gene was constructed by removing signal sequence from p19CSFm(see: Figure 5). To clone the mature hG-CSF gene, Primer 7: 5'-GCGAATTCATATGACCCCCCTGGGCCCTGCCAGC-3'(SEQ ID NO: 8) was prepared. Employing primer 2 and primer 7, p19CSFm was subjected to PCR amplification, and the amplified DNA molecules were digested with two restriction enzymes, *Nde*I and *Bam*HI. The resultant DNA fragment was joined into the T7 promoter-containing plasmid pET21c(Novagen Co., U.S.A.) digested with *Nde*I and *Bam*HI. After transformation of E. coli XL1-Blue with the ligated product by electroporation, the transformants were selected on the LB agar medium containing ampicillin(50 μg/ℓ) and the recombinant plasmid pEDCSFm was obtained therefrom(see: Figure 4).

For production of hG-CSF protein in *E. coli*, the recombinant plasmid pEDCSFm was introduced into *E. coli* BL21(DE3)(*F- ompT hsdSB(rB- mB-) gal dcm (DE3)* a prophage carrying the T7 RNA polymerase gene). The transformed *E. coli* harboring the recombinant plasmid was inoculated into 50ml of liquid LB medium in a 250ml flask and incubated at 37℃. When the cell concentration reached to OD600 of about 0.7, 1mM IPTG(isopropyl-β-thiogalactoside) was added to the culture to induce the expression of hG-CSF gene. After 4 hour induction, 1ml of the culture broth was aliquoted. The aliquoted broth was centrifuged at a speed

of 6,000rpm for 5min at 4℃ to collect cells, which were then washed, centrifuged again at a speed of 6,000rpm for 5min at 4℃, and then resuspended in 0.2ml of TE buffer. The mixture of 64 ㎕ aliquot of cell suspension and 16 ㎕ of sample buffer(Tris-HCl 60mM: 25% glycerol (v/v): 2% SDS(v/v): 2-mercaptoethanol 14.4mM: 0.1% bromophenol blue) was heated at 100℃ for 10min, and then subjected to SDS-PAGE(sodium-dodecyl sulfate polyacrylamide gel electrophoresis) in separating gel. After SDS-PAGE, the gel was stained in a staining solution(Coomassie brilliant blue R 0.25g/ℓ : methanol 40%(v/v), acetic acid 7%(v/v)) for 2 hours, and destained two times in destaining solution(methanol 40%(v/v), acetic acid 7%(v/v)) for 2 hours each time. The SDS-PAGE did not show the protein band corresponding to hG-CSF protein.

Example 3: Construction of the recombinant plasmid, pEDCSFmII

Based on the report by Devlin et al. that high G+C content in N-terminal portion of hG-CSF gene has inhibitory effect on transcription and translation of the gene(see: Devlin et al., *Gene*, 65:13-22 (1988)), primer 8: 5'-GCGAATTCATATGACTCCGTTAGGTCCAGCCAGC-3'(SEQ ID NO: 9) was prepared to obtain the gene which has lower G+C content such that the gene can be transcribed and translated efficiently in *E. coli*.

Plasmid p19CSFm was amplified by PCR using primer 8 and primer 2. The resulting PCR product was digested with *Nde*I and *Bam*HI, cloned into the same site of pET21c, and then transformed into *E. coli* XL1-Blue. The transformed *E. coli* XL1-Blue were selected on the LB agar medium containing ampicillin(50 ㎍/ℓ) and the recombinant plasmid pEDCSFmII was obtained therefrom(see: Figure 6). Comparing with the sequence of the fragment in pEDCSFm, the nucleotide sequence of the fragment in pEDCSFmII was found to be identical in N-terminal portion of the gene,

meanwhile, be different in 6 nucleotide residues overall, which give rise the changes in 5 codons(ACC CCC CTG GGC CCT → ACT CCG TTA GGT CCA).

Plasmid pEDCSFmII was transformed into *E. coli* BL21(DE3) and its expression was analyzed. Culturing of transformed *E. coli*, and production and analysis of total protein were carried out as described in Example 2, and fractionation of insoluble aggregate was performed as follows. Cells obtained by centrifugation of 1ml of culture broth were suspended in 0.5ml of TE buffer(Tris-HCl 10mM, EDTA 1mM, pH 8.0), and then centrifuged again at a speed of 3,000xg for 5min at 4℃ followed by resuspension in 0.2ml of TE buffer. Cells were disrupted by using a ultrasonicator(Branson Ultrsonics Co., U.S.A.) and then centrifuged at a speed of 10,000xg for 10min at 4℃. The supernatant was classified as soluble proteins and the pellet was dissolved in 0.2ml of TE buffer and classified as insoluble proteins. The content of hG-CSF protein expressed in *E. coli* BL21(DE3) transformed with plasmid pEDCSFmII was identified to be as much as about 40% of total protein, and, most of hG-CSF protein produced was found to be insoluble aggregates with no biological activity.

Example 4: Construction of the recombinant plasmid, pTrcSCSFmII

The signal sequence of endoxylanase derived from *Bacillus* sp. was used for the secretion of hG-CSF protein from *E. coli*. To join the signal sequence of endoxylanase to the N-terminal of hG-CSF polypeptide by PCR, primer 9: 5'-GGAATTCACATGTTTAAG TTTAAAAAGAAATTC-3'(SEQ ID NO: 10), primer 10: 5'-GGCTGGACCTAACGGAGTTGCAGAGGCGG-3'(SEQ ID NO: 11) and primer 11: 5'-GCAACCGCCTCTGCAACTCCGTTAGGTCCAGCC-3'(SEQ ID NO: 12) were prepared, respectively. From PCR performed using primers 9 and 10, and endoxylanase gene-

carrying plasmid pKJX4(see: Jeong et al., *Enzyme Microb Technol.* 22(7):599-605(1998)) as a target DNA, PCR product containing endoxylanase signal sequence was obtained, and from PCR performed using primers 2 and 11, PCR product containing hG-CSF gene was obtained. Again, PCR was performed using two PCR products obtained above, and primers 2 and 9 to obtain PCR product containing endoxylanase signal sequence-fused hG-CSF gene, which was then digested with two restriction enzymes, *Afl*III and *Bam*HI. Also, the plasmid pTrc99A(Pharmacia Biotech Co., U.S.A.) carrying a strong inducible promoter, trc promoter, was digested with *Nco*I and *Bam*HI, and then ligated with the above two PCR products. The ligation mixture was transformed into *E. coli* XL1-Blue. The transformants were selected on the LB agar medium containing ampicillin(50 μg/ℓ) and the recombinant plasmid pTrcSCSFmII was obtained therefrom(see: Figure 8). The nucleotide sequence of the N-terminal portion of hG-CSF gene was determined using an automatic DNA sequencer(ABI Prism model 377, Perkin Elmer Co., U.S.A.)(see: Figure 9).

In order to analyze the secretion of hG-CSF protein in various strains of *E. coli*, the recombinant plasmid pTrcSCSFmII was transformed into *E. coli* BL21(DE3), *E. coli* HB101(*F- hsdS20(rk-, mk-) recA13 ara-14 proA2 lacY1 galK2 rpsL20(strr) xyl1-5 mtl-1 supE44* λ -), *E. coli* MC4100(*F- araD139* Δ *(argF-lac)U169 rpsL150(strr) relA1 flbB5301 deoC1 ptsF25 rbsR*) and *E. coli* W3110(derived from *E. coli* K-12, λ -, F-, prototrophic). Each transformant was inoculated into 50ml aliquots of LB medium containing ampicillin(50 μg/ℓ), respectively, to perform the experiments for measuring expression level and protein analysis by the method described in Example 2. After induction with 1mM IPTG, however, the cells began to lyse abruptly, and within 2 hours most cells were lysed. The cultures were centrifuged to obtain transformed *E. coli* cells and proteins were analyzed using SDS-PAGE to find out no protein corresponding hG-CSF protein was obtained.

Example 5: Construction of the recombinant plasmid, pTrcKCSFmII

Considering the fact that ampicillin resistance gene(Apr) in plasmid pTrcSCSFmII is coding for β -lactamase which is secreted to the periplasm of *E. coli*, it may be conjectured that there was a competition between β -lactamase and hG-CSF protein for secretion, and such competition brought about cell lysis observed in Example 4. Based on such hypothesis, kanamycin resistance gene(Kmr) was employed instead of ampicillin resistance gene(Apr) as a selection marker since kanamycin resistance gene encodes the non-secreted protein, still can be used as a selection pressure. In order to substitute ampicillin resistance gene(Apr) in plasmid pTrcSCSFmII with kanamycin resistance gene(Kmr), PCR was performed using primer 12: 5'-GCGAATTCTTTAAAGCCACGTTGTGTCCTCAAA-3'(SEQ ID NO: 13) and peimer 13: 5'-GCGAATTCTTTAAATTAGAAAAACTCATCGAGCATC-3'(SEQ ID NO: 14), and plasmid pACYC177 as a target DNA. The resulting PCR product containing kanamycin resistance gene was digested with *Dra*I, and then ligated to the plasmid pTrcSCSFmII which was partially digested with *Dra*I to remove ampicillin resistance gene. The ligation product was transformed into *E. coli* XL1-Blue. The transformants were selected on LB agar medium containing kanamycin(25 μg/ℓ) and the recombinant plasmid pTrcKCSFmII was obtained therefrom(see: Figure 10).

To analyze the secretion of hG-CSF protein, the recombinant plasmid pTrcKCSFmII was transformed into *E. coli* BL21(DE3), *E. coli* HB101, *E. coli* MC4100 and *E. coli* W3110. Each transformant was inoculated into 50ml aliquots of LB medium containing kanamycin(25 μg/ℓ), respectively, to perform the experiments for measuring expression level and protein analysis by the method described in Example 2, and same result was obtained as in Example 4 wherein

pTrcSCSFmII was used. That is, with pTrcKCSFmII also, cells were lysed excessively upon induction with 1mM IPTG and SDS-PAGE analysis of recovered protein showed no protein corresponding to hG-CSF protein. From the above studies, it has been found that the substitution of ampicillin resistance gene with kanamycin resistance gene exert no effect on avoiding cell lysis, therefore, another genetic manipulation which can solve the intrinsic problem of cell lysis caused by secretion of hG-CSF protein is necessary.

Example 6: Construction of the recombinant plasmids, pTHSCSFmII and pTHKCSFmII

To facilitate *E. coli* to secrete hG-CSF protein without resulting in cell lysis, the strategy of inserting a small oligopeptide between endoxylanase signal sequence and hG-CSF protein was employed. For this purpose, PCR was performed using primer 14: 5'-CACCATCACCATATCGAAGGCCGTACTCCGTTAGGTCCA-3'(SEQ ID NO: 15) and primer 15: 5'-GATATGGTGATGGTGATGGTGCGGGCCAGCTGCAGAGGCGG-3'(SEQ ID NO: 16), and pEDSCSFmII as a target DNA. First, PCR with primers 14 and 2, and PCR with primers 15 and 9 were performed, respectively. Then, the two PCR products were mixed to be subjected to another PCR using primers 2 and 9, and the resulting product was digested with *Afl*III and *Bam*HI to join into the *Nco*I/*Bam*HI site of plasmid pTrc99A. After introducing the ligation product into *E. coli* XL1-Blue, the transformants were selected on a LB agar medium containing ampicillin(50 $\mu g/\ell$), and the recombinant plasmid pTHSCSFmII was obtained therefrom(see: Figure 11). Figure 12 shows the nucleotide sequence and deduced amino acid sequence of the inserted DNA in the plasmid pTHSCSFmII, wherein the italic indicates the endoxylanase signal sequence and the bold indicates inserted oligopeptide sequence.

As similarly as in Example 5, in order to substitute

ampicillin resistance gene(Apr) with kanamycin resistance gene(Kmr), the plasmid pTHSCSFmII was partial digested with DraI to remove ampicillin resistance gene(Apr) and then ligated with DNA fragment carrying kanamycin resistance gene obtained by digestion of pTrcKCSFmII with DraI. The ligation product was transformed into *E. coli* XL1-Blue. The transformants were selected on LB agar medium containing kanamycin(50 $\mu g/\ell$) and the recombinant plasmid pTHKCSFmII was obtained therefrom(see: Figure 13).

Example 7: Secretion of hG-CSF fusion protein from the transformed E. coli carrying the recombinant plasmid pTHKCSFmII

To analyze the expression and secretion of hG-CSF fusion protein from pTHKCSFmII, the recombinant plasmid pTHKCSFmII was transformed into *E. coli* BL21(DE3), *E. coli* HB101, *E. coli* MC4100 and *E. coli* W3110, respectively. Each transformant was cultured in 50ml aliquots of LB medium under temperatures of 37℃ and 30℃, respectively, followed by analyses of secretion of the hG-CSF fusion protein as described in Example 2. After 4 hour induction of hG-CSF gene expression with 1mM IPTG, 1ml aliquots of culture broth were centrifuged to obtain the cells, which were then analyzed by SDS-PAGE for the expression of hG-CSF fusion protein to show the secretion of hG-CSF fusion protein from all the transformed *E. coli*. The contents and the secretion efficiencies of hG-CSF fusion protein secreted from the each transformed *E. coli* are shown in Table 1.

Table 1: Comparison of production and secretion of hG-CSF in recombinant *E. coli*

Host Cell	Content of hG-CSF(%) in Total Protein		Secretion of hG-CSF(%)	
Culture Time	30°C	37°C	30°C	37°C
E. coli BL21(DE3)	22.7	22.1	>98	>98
E. coli HB101	13.5	12.8	81	75

E. coli MC4100	22.1	20.8	>98	>98
E. coli W3110	10.5	10.0	77	66
E. coli XL1-Blue	9.4	8.8	56	51

To examine if the secreted hG-CSF protein has been processed correctly, i.e., if the signal sequence has been removed correctly, hG-CSF fusion protein was isolated from the gel and its N-terminal amino acid sequence was determined to be N'-Ala-Gly-Pro-His-His-His-His-His-His-Ile-Glu-Gly-Arg-Thr-C', which is in agreement with the deduced amino acid sequence of N-terminal portion of hG-CSF fusion protein, indicating that the hG-CSF fusion protein was successfully secreted from *E. coli*. Of two temperature conditions, all the transformants showed higher secretion efficiency and higher hG-CSF protein content at 30℃ than 37℃. Of 5 strains of transformed *E. coli*, BL21(DE3) and MC4100 showed the highest production yield. Thus, *E. coli* MC4100 transformed with the recombinant plasmid pTHKCSFmII was named *E. coli* MC4100/pTHKCSFmII, which was deposited with the Korean Collection for Type Cultures(KCTC) affiliated to Korea Research Institute of Bioscience and Biotechnology(KRIBB), an international depository authority, under accession(deposition) No. KCTC 0754BP on Mar. 13, 2000.

The hG-CSF fusion polypeptide expressed in the transformed *E. coli* MC4100/pTHKCSFmII was processed during the secretion process to the periplasm through innermembrane to remove endoxylanase secretion sequence, resulting in secretion of oligopeptide/hG-CSF fusion protein. The hG-CSF fusion protein can be isolated easily by using a nickel column due to 6 histidine residues in N-terminal portion of the fusion protein, and C-terminus of the oliopeptide in the fusion protein can be recognized by Factor Xa and the oligopeptide is cleaved off to give a complete hG-CSF protein.

As clearly illustrated and demonstrated above, the

present invention provides an *E. coli* producing and secreting human granulocyte-colony stimulating factor(hG-CSF), a plasmid vector therefor, and a process for producing complete hG-CSF protein with high purity from the protein pool secreted by the said microorganism. In accordance with the invention, the hG-CSF protein can be prepared with high purity through rather simple process facilitating secretion of large amount of hG-CSF fusion protein into the periplasm, which does not require complicated processes such as solubilization and subsequent refolding required for isolation of the hG-CSF protein produced in cytoplasm as insoluble inclusion bodies by conventional techniques, thus, the hG-CSF protein can be widely used as an active ingredient in the development of supplementary agents for anticancer therapy.

It will be understood that the above description is merely illustrative of the preferred embodiment and it is not intended to limit the scope of the invention to the particular forms set forth, but on the contrary, it is intended to cover such alteranatives, modifications and equivalents as may be included within the spirit and scope of the invention as defined by the claims.

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in description
On page 7 , lines 18-25 .

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on additional sheet ☐

Name of depositary institution

Korean Collection for Type Cultures (KCTC)

Address of depositary institution (including postal code and country)

Korea Research Institute of Bioscience and Biotechnology(KRIBB)
#52, Oun-dong, Yusong-ku,
Taejon 305-333,
Republic of Korea

Date of deposit	Accession Number
Mar. 13, 2000	KCTC 0754BP

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information continues on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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WHAT IS CLAIMED IS:

1. A recombinant plasmid vector which comprises:

a kanamycin resistance gene;

a promoter;

an endoxylanase signal sequence;

a nucleotide sequence coding for an oligopeptide consisting of 13 amino acids including 6 consecutive histidine residues; and,

a human granulocyte colony stimulating factor(hG-CSF) gene.

2. The recombinant plasmid vector of claim 1, wherein the oligopeptide has an amino acid sequence of isoleucine-glutamatic acid-glycine-arginine(Ile-Glu-Gly-Arg) at the C-terminus.

3. A recombinant plasmid vector, pTHKCSFmII represented in Figure 13 which comprises:

a kanamycin resistance gene;

a Trc promoter;

an endoxylanase signal sequence derived from *Bacillus* sp.;

a nucleotide sequence coding for an oligopeptide of SEQ ID NO: 1; and,

a modified gene coding for a human granulocyte colony stimulating factor(hG-CSF), which includes a nucleotide sequence of SEQ ID NO: 26 at the N-terminus.

4. A microorganism, *E. coli* transformed with the plasmid vector, pTHKCSFmII of claim 3.

5. The microorganism of claim 4, wherein the *E. coli*

is selected from the group consisting of *E. coli* XL1-Blue, *E. coli* MC4100, *E. coli* BL21(DE3), *E. coli* HB101 and *E. coli* W3110.

6. *E. coli* MC4100/pTHKCSFmII(KCTC 0754BP) transformed with the plasmid vector, pTHKCSFmII of claim 3.

7. A process for preparing a human granulocyte colony stimulating factor, which comprises the steps of:

culturing *E. coli* transformed with the plasmid vector of claim 1 to obtain a human granulocyte colony stimulating factor fusion protein; and,

treating the human granulocyte colony stimulating factor fusion protein with a protease to obtain a human granulocyte colony stimulating factor.

8. The process for preparing a human granulocyte colony stimulating factor of claim 7, wherein the plasmid vector of claim 1 is pTHKCSFmII.

9. The process for preparing a human granulocyte colony stimulating factor of claim 7, wherein the human granulocyte colony stimulating factor fusion protein is obtained from the culture by employing Ni-column.

10. The process for preparing a human granulocyte colony stimulating factor of claim 7, wherein the protease is Factor Xa.

Fig. 1

```
  1 ATG GCT GGA CCT GCC ACC CAG AGC CCC ATG AAG CTG ATG GCC CTG  45
 46 CAG CTG CTG CTG TGG AGT GCA CTC TGG ACA GTG CAG GAA GCC ACC  90
 91 CCC CTG GGC CCT GCC AGC TCC CTG CCC CAG AGC TTC CTG CTC AAG 135
136 TGC TTA GAG CAA GTG AGG AAG ATC CAG GGC GAT GGC GCA GCG CTC 180
181 CAG GAG AAG CTG GCA GGC TGC TTG AGC CAA CTC CAT AGC GGC CTT 225
226 TTC CTC TAC CAG GGG CTC CTG CAG GCC CTG GAA GGG ATC TCC CCC 270
271 GAG TTG GGT CCC ACC TTG GAC ACA CTG CAG CTG GAC GTC GCC GAC 315
316 TTT GCC ACC ACC ATC TGG CAG CAG ATG GAA GAA CTG GGA ATG GCC 360
361 CCT GCC CTG CAG CCC ACC CAG GGT GCC ATG CCG GCC TTC GCC TCT 405
406 GCT TTC CAG CGC CGG GCA GGA GGG GTC CTA GTT GCC TCC CAT CTG 450
451 CAG AGC TTC CTG GAG GTG TCG TAC CGC GTT CTA CGC CAC CTT GCC 495
496 CAG CCC TAA TAA                                           508
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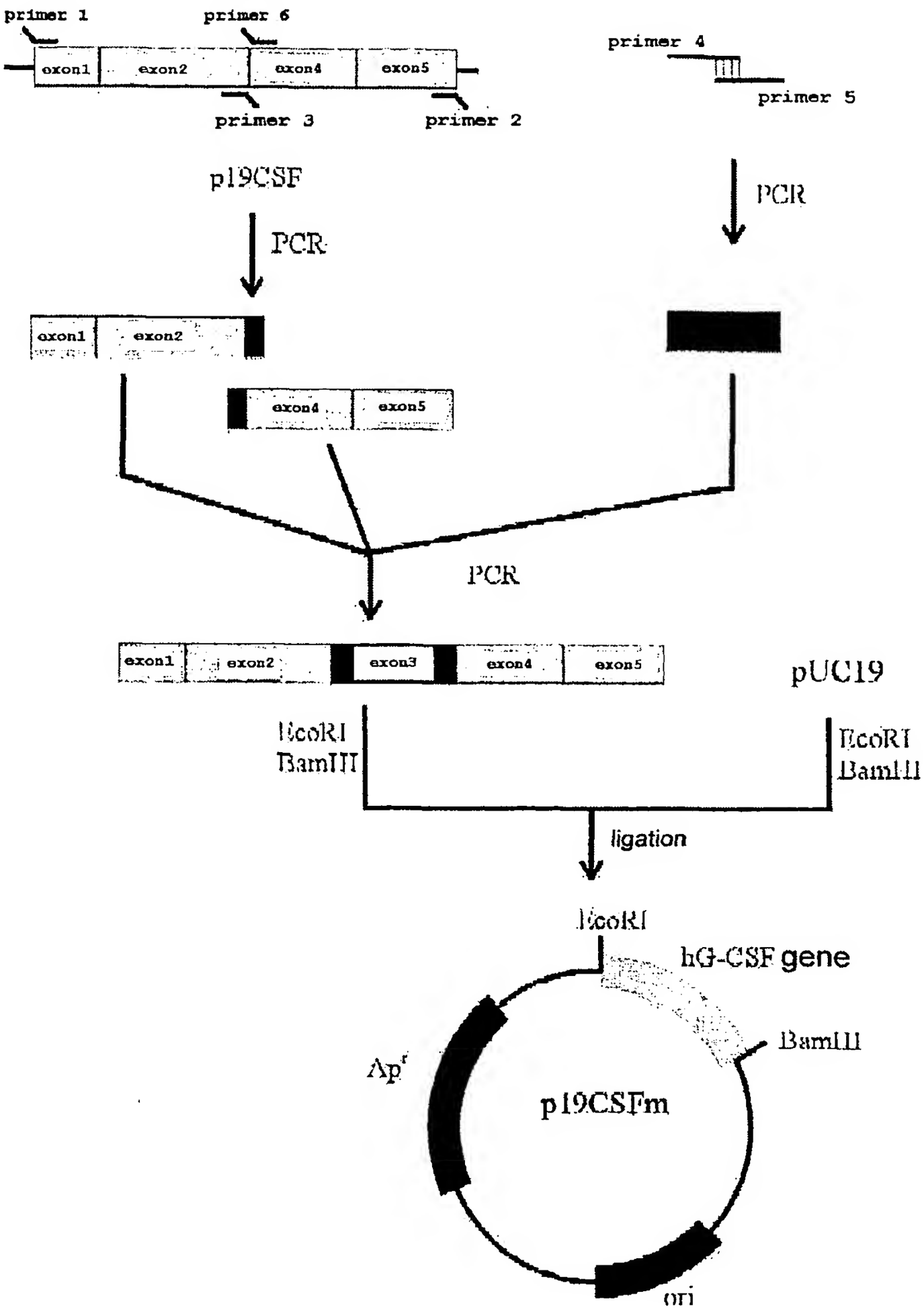
stop codon (see: SEQ ID NO: 17)

Fig. 2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exon2
exon3
exon4
exon5
PCR
pUC19
EcoRI
BamIII
EcoRI
BamIII
ligation
EcoRI
hG-CSF gene
BamIII
Ap^r
p19CSFm
ori

Fig. 3

```
    -30                                                                 -16
  1 ATG GCT GGA CCT GCC ACC CAG AGC CCC ATG AAG CTG ATG GCC CTG  45

    -15                                                          -1   +1
 46 CAG CTG CTG CTG TGG AGT GCA CTC TGG ACA GTG CAG GAA GCC ACC  90
                                                              Thr

    2                                                                   16
 91 CCC CTG GGC CCT GCC AGC TCC CTG CCC CAG AGC TTC CTG CTC AAG 135
    Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys

    17                                                                  31
136 TGC TTA GAG CAA GTG AGG AAG ATC CAG GGC GAT GGC GCA GCG CTC 180
    Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu

    32                                                                  46
181 CAG GAG AAG CTG TGT GCC ACC TAC AAG CTG TGC CAC CCC GAG GAG 225
    Gln Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu

    47                                                                  61
226 CTG GTG CTG CTC GGA CAC TCT CTG GGC ATC CCC TGG GCT CCC CTG 270
    Leu Val Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu

    62                                                                  76
271 AGC AGC TGC CCC AGC CAG GCC CTG CAG CTG GCA GGC TGC TTG AGC 315
    Ser Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser

    77                                                                  91
316 CAA CTC CAT AGC GGC CTT TTC CTC TAC CAG GGG CTC CTG CAG GCC 360
    Gln Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala

    92                                                                 106
361 CTG GAA GGG ATC TCC CCC GAG TTG GGT CCC ACC TTG GAC ACA CTG 405
    Leu Glu Gly Ile Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu

    107                                                                121
406 CAG CTG GAC GTC GCC GAC TTT GCC ACC ACC ATC TGG CAG CAG ATG 450
    Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Met

    122                                                                136
451 GAA GAA CTG GGA ATG GCC CCT GCC CTG CAG CCC ACC CAG GGT GCC 495
    Glu Glu Leu Gly Met Ala Pro Ala Leu Gln Pro Thr Gln Gly Ala

    137                                                                151
496 ATG CCG GCC TTC GCC TCT GCT TTC CAG CGC CGG GCA GGA GGG GTC 540
    Met Pro Ala Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly Val

    152                                                                166
541 CTA GTT GCC TCC CAT CTG CAG AGC TTC CTG GAG GTG TCG TAC CGC 585
    Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr Arg

    167                                 174
586 GTT CTA CGC CAC CTT GCC CAG CCC TAA TAA                     616
    Val Leu Arg His Leu Ala Gln Pro stop codon
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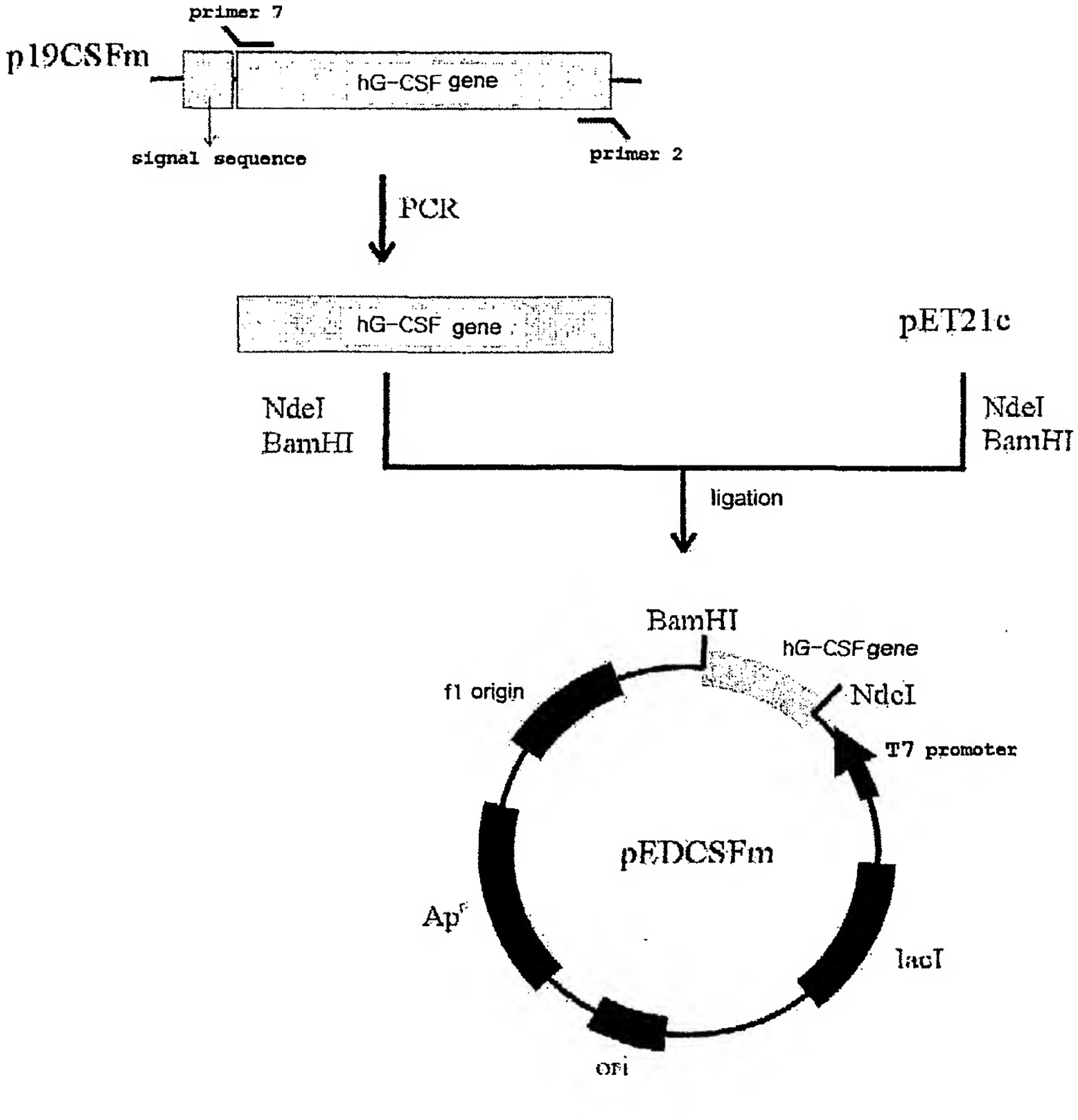
(see: SEQ ID NO: 18)

(see: SEQ ID NO: 19)

Fig. 4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signal sequence
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BamHI
NdeI
BamHI
ligation
BamHI
hG-CSFgene
f1 origin
NdeI
T7 promoter
pEDCSFm
Ap
lacI
ori

Fig. 5

```
    1                                                              15
 1  ATG ACC CCC CTG GGC CCT GCC AGC TCC CTG CCC CAG AGC TTC CTG   45
    Met Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu

   16                                                              30
46  CTC AAG TGC TTA GAG CAA GTG AGG AAG ATC CAG GGC GAT GGC GCA   90
    Leu Lys Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala

   31                                                              45
91  GCG CTC CAG GAG AAG CTG TGT GCC ACC TAC AAG CTG TGC CAC CCC 135
    Ala Leu Gln Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro

   46                                                              60
136 GAG GAG CTG GTG CTG CTC GGA CAC TCT CTG GGC ATC CCC TGG GCT 180
    Glu Glu Leu Val Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala

   61                                                              75
181 CCC CTG AGC AGC TGC CCC AGC CAG GCC CTG CAG CTG GCA GGC TGC 225
    Pro Leu Ser Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys

   76                                                              90
226 TTG AGC CAA CTC CAT AGC GGC CTT TTC CTC TAC CAG GGG CTC CTG 270
    Leu Ser Gln Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu

   91                                                             105
271 CAG GCC CTG GAA GGG ATC TCC CCC GAG TTG GGT CCC ACC TTG GAC 315
    Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu Gly Pro Thr Leu Asp

  106                                                             120
316 ACA CTG CAG CTG GAC GTC GCC GAC TTT GCC ACC ACC ATC TGG CAG 360
    Thr Leu Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile Trp Gln

  121                                                             135
361 CAG ATG GAA GAA CTG GGA ATG GCC CCT GCC CTG CAG CCC ACC CAG 405
    Gln Met Glu Glu Leu Gly Met Ala Pro Ala Leu Gln Pro Thr Gln

  136                                                             150
406 GGT GCC ATG CCG GCC TTC GCC TCT GCT TTC CAG CGC CGG GCA GGA 450
    Gly Ala Met Pro Ala Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly

  151                                                             165
451 GGG GTC CTA GTT GCC TCC CAT CTG CAG AGC TTC CTG GAG GTG TCG 495
    Gly Val Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu Val Ser

  166                                                  175
496 TAC CGC GTT CTA CGC CAC CTT GCC CAG CCC TAA TAA          531
    Tyr Arg Val Leu Arg His Leu Ala Gln Pro stop codon
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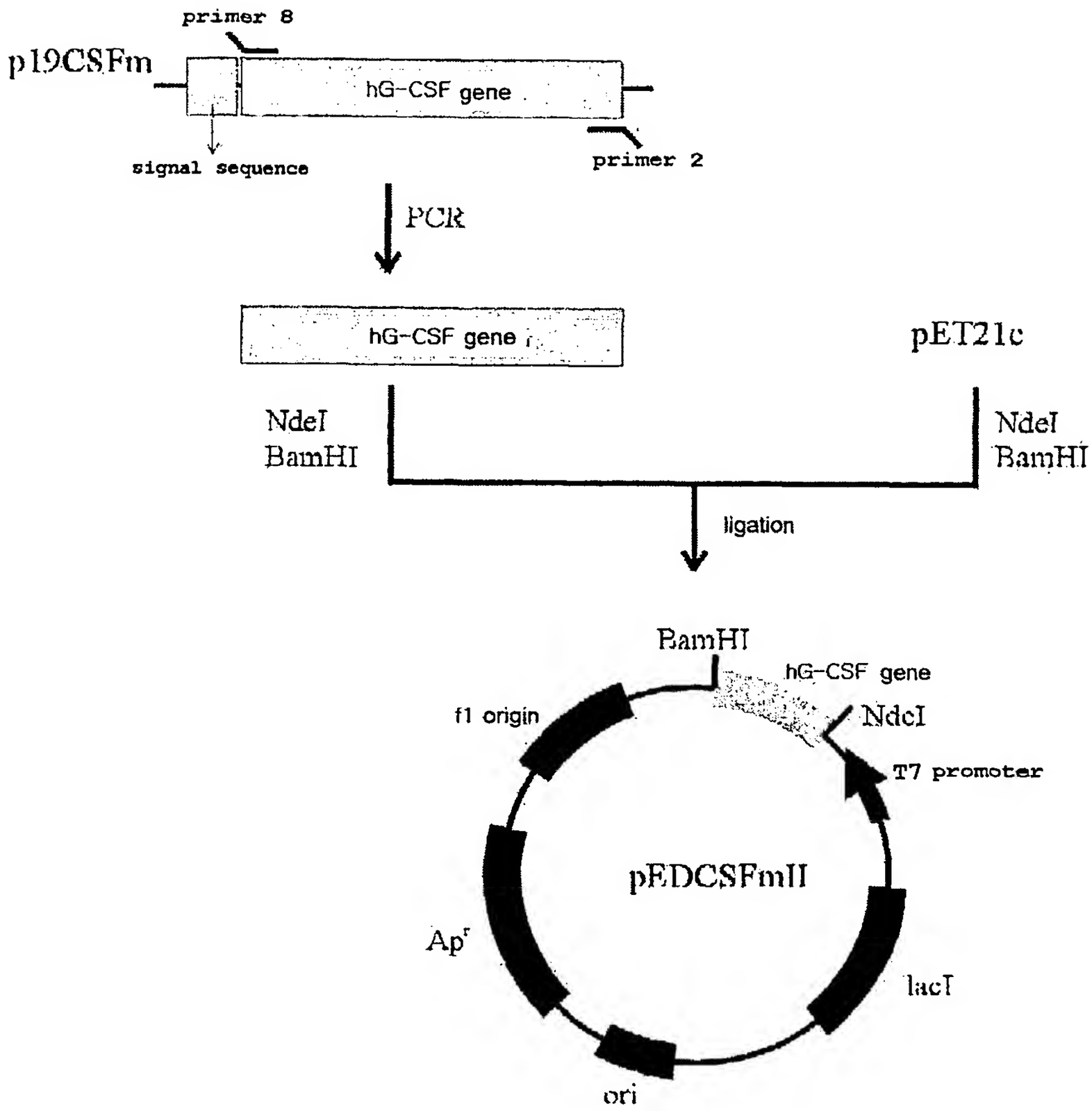
(see: SEQ ID NO: 20)
(see: SEQ ID NO: 21)

Fig. 6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BamHI
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BamHI
ligation
BamHI
hG-CSF gene
f1 origin
NdeI
T7 promoter
pEDCSFmII
Ap
lacI
ori

Fig. 7

```
  1                                                              15
1 ATG ACT CCG TTA GGT CCA GCC AGC TCC CTG CCC CAG AGC TTC CTG  45
  Met Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu
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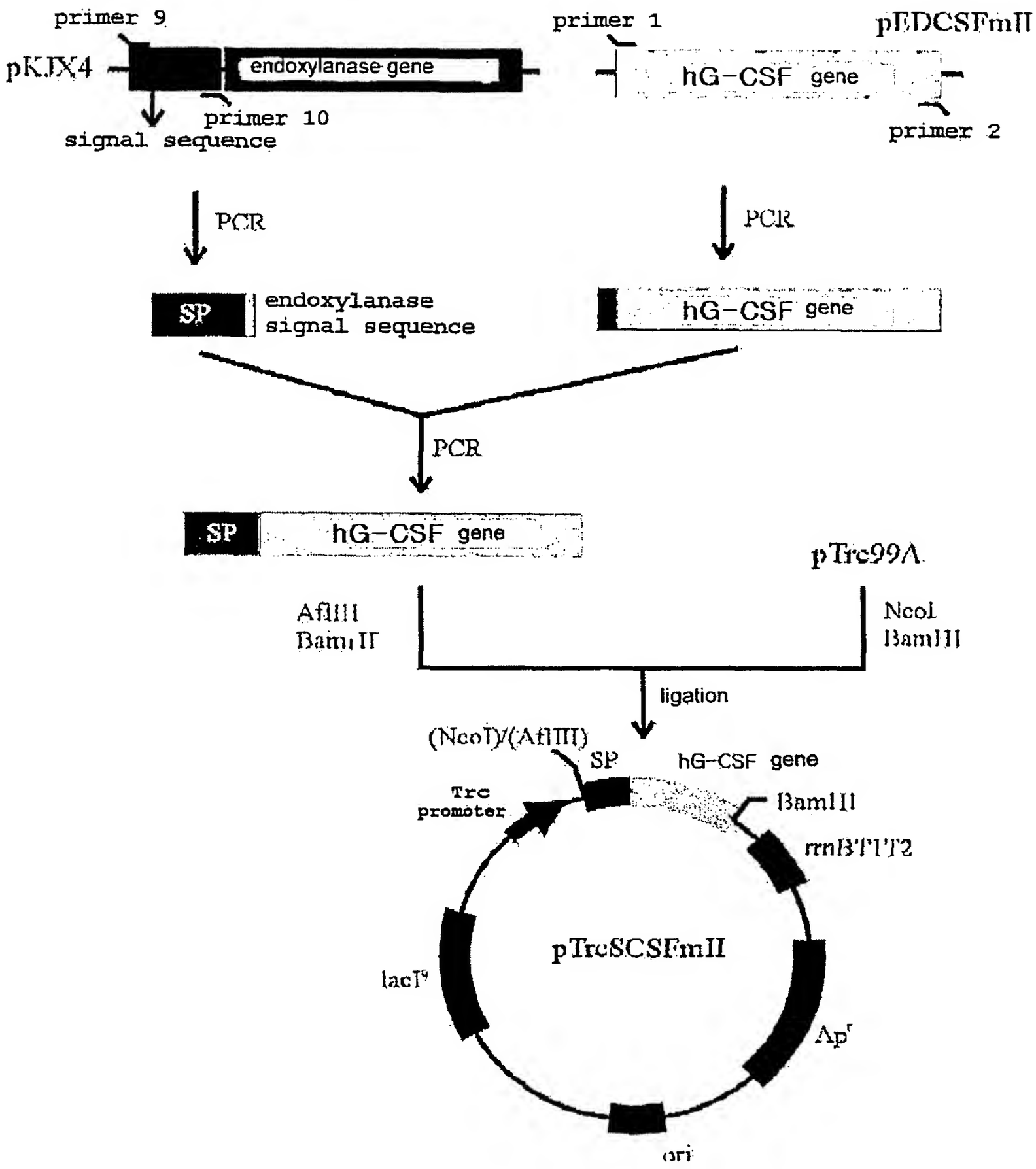
(see: SEQ ID NO: 22)

(see: SEQ ID NO: 23)

Fig. 8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primer 2
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SP
endoxylanase
signal sequence
hG-CSF gene
PCR
SP
hG-CSF gene
pTrc99A
AflIII
BamHI
NcoI
BamHI
ligation
(NcoI)/(AflIII)
SP
hG-CSF gene
Trc
promoter
BamHI
rrnBT1T2
pTrcSCSFmII
lacI^q
Ap^r
ori

Fig. 9

```
   -28                                                                -14
 1 ATG TTT AAG TTT AAA AAG AAA TTC TTA GTG GGA TTA ACG GCA GCT  45
   Met Phe Lys Phe Lys Lys Lys Phe Leu Val Gly Leu Thr Ala Ala

   -13                                                    -1  +1   2
46 TTC ATG AGT ATC AGC ATG TTT TCT GCA ACC GCC TCT GCA ACT CCG  90
   Phe Met Ser Ile Ser Met Phe Ser Ala Thr Ala Ser Ala Thr Pro

    3                                                              17
91 TTA GGT CCA GCC AGC TCC CTG CCC CAG AGC TTC CTG CTC AAG TGC 135
   Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys Cys
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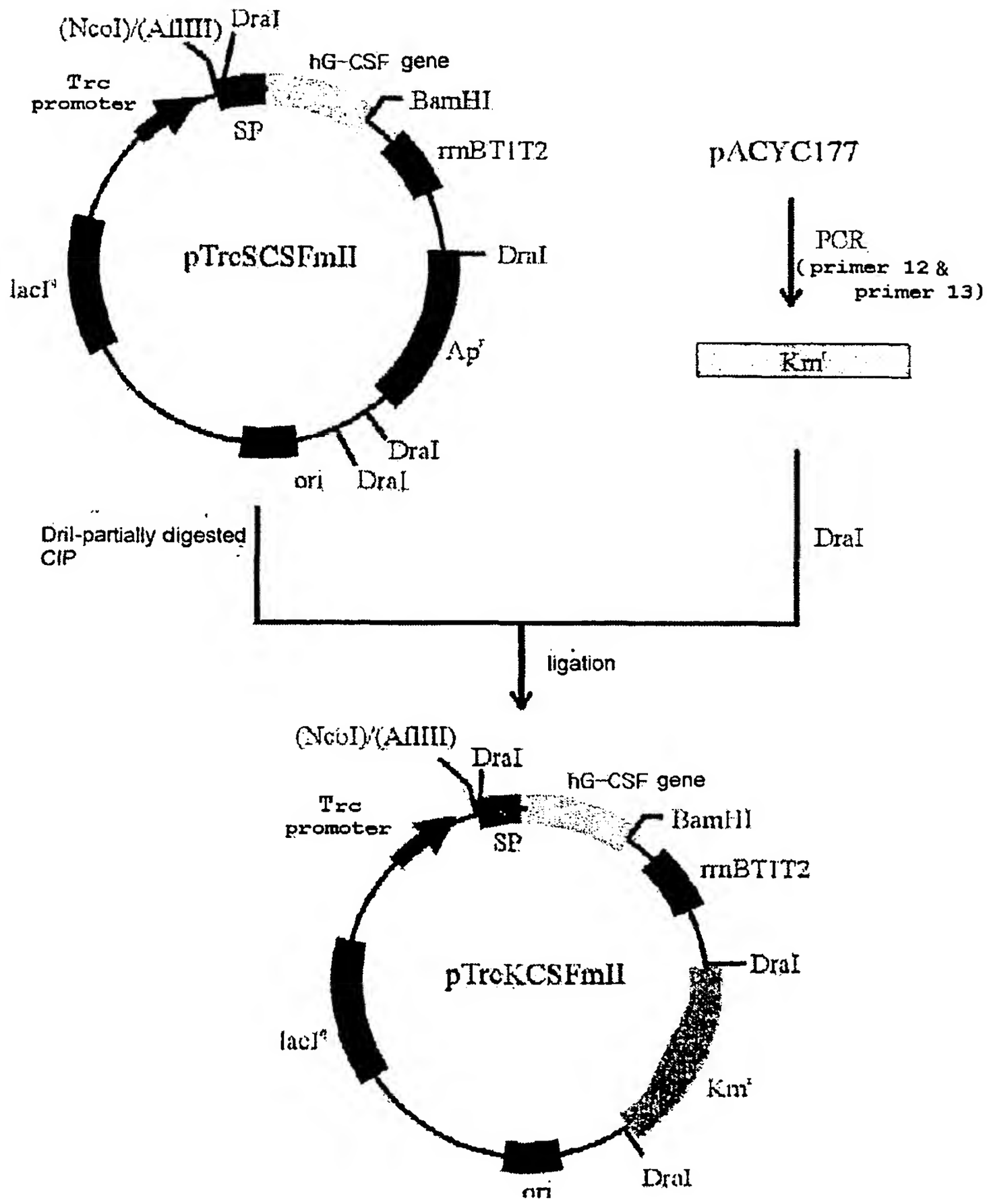
(see: SEQ ID NO: 24)
(see: SEQ ID NO: 25)

Fig. 10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Trc promoter
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SP
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lacI^q
Ap^r
DraI
ori
DraI
DraI-partially digested CIP
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PCR (primer 12 & primer 13)
Km^r
DraI
ligation
(NcoI)/(AflIII)
DraI
hG-CSF gene
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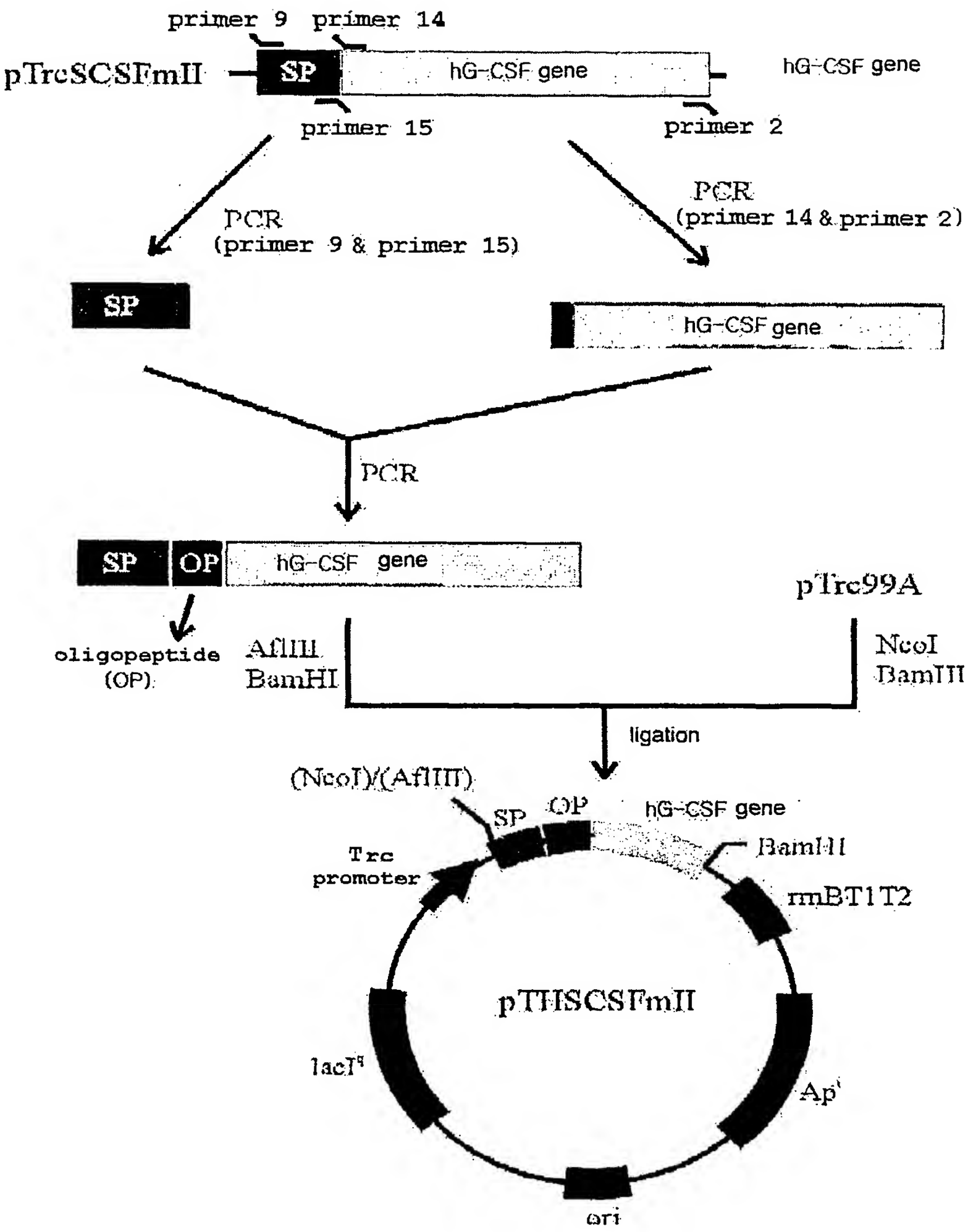
Fig. 11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SP
OP
hG-CSF gene
oligopeptide
(OP)
AflIII
BamHI
pTrc99A
NcoI
BamHI
ligation
(NcoI)/(AflIII)
SP
OP
hG-CSF gene
Trc
promoter
BamHI
rrnBT1T2
pTHSCSFmII
lacI^q
Ap^r
ori

Fig. 12

```
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 1  ATG TTT AAG TTT AAA AAG AAA TTC TTA GTG GGA TTA ACG GCA GCT  45
    Met Phe Lys Phe Lys Lys Lys Phe Leu Val Gly Leu Thr Ala Ala

    -13                                                    -1  +1   2
46  TTC ATG AGT ATC AGC ATG TTT TCT GCA ACC GCC TCT GCA GCT GGC  90
    Phe Met Ser Ile Ser Met Phe Ser Ala Thr Ala Ser Ala Ala Gly

     3                                                              17
91  CCG CAC CAT CAC CAT CAC CAT ATC GAG GGA AGG ACT CCG TTA GGT 135
    Pro His His His His His His Ile Glu Gly Arg Thr Pro Leu Gly

    18                                                              32
136 CCA GCC AGC TCC CTG CCC CAG AGC TTC CTG CTC AAG TGC TTA GAG 180
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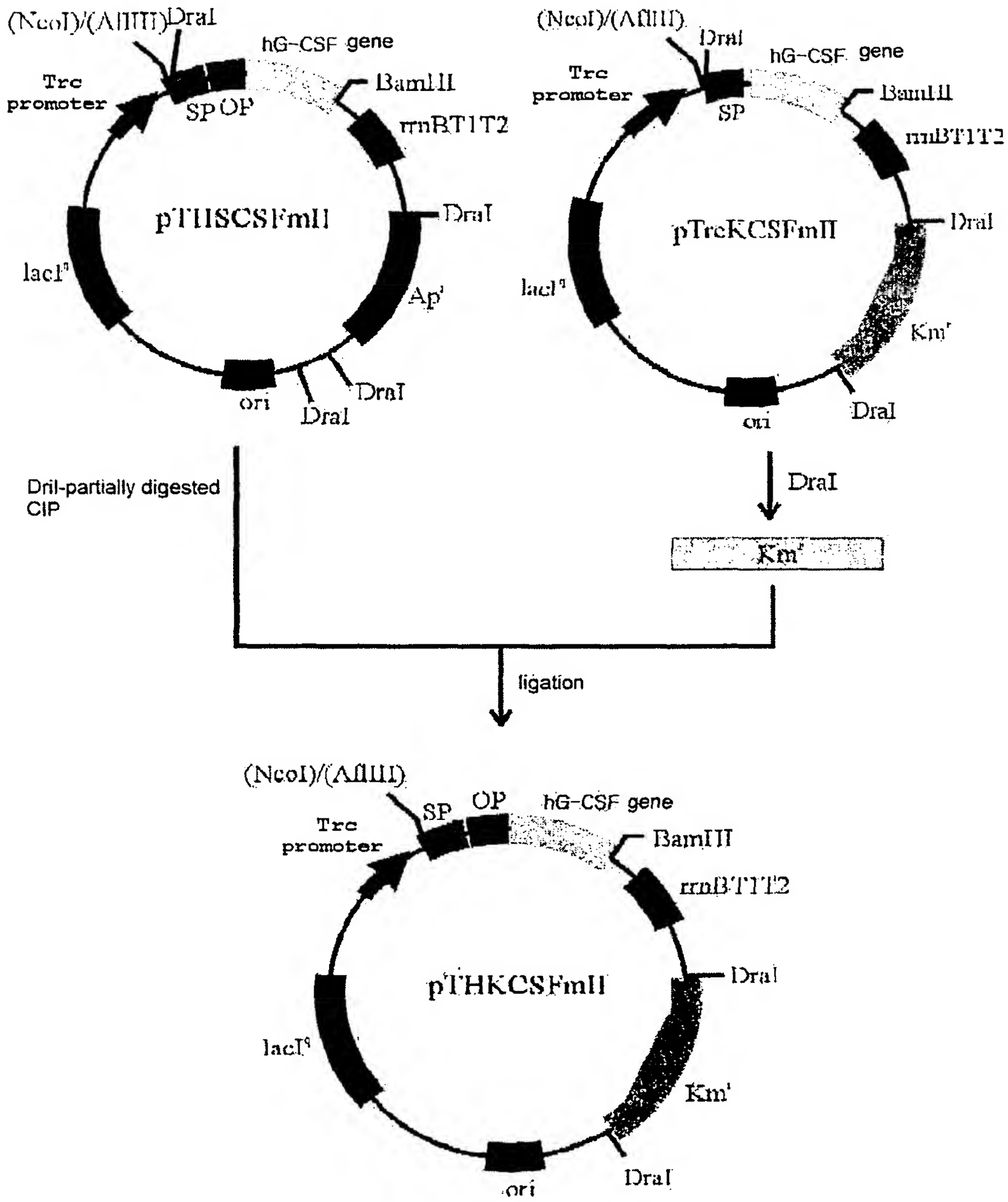
(see: SEQ ID NO: 26)

(see: SEQ ID NO: 27)

Fig. 13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Trc promoter
SP
OP
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pTHSCSFmII
DraI
lacI^q
Ap^r
ori
DraI
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Km^r
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ligation
SP
OP
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Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu Val
35 40 45

Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys
50 55 60

Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser
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Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser
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Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala Asp
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115 120 125

Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe
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35 40 45

Val Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser

50 55 60

Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His
65 70 75 80

Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile
85 90 95

Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala
100 105 110

Asp Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala
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Pro Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala
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<400> 27
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1 5 10 15

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20 25 30

His His His His His Ile Glu Gly Arg Thr Pro Leu Gly Pro Ala Ser
35 40 45

Ser Leu Pro Gln Ser Phe Leu Leu Lys Cys Leu Glu
50 55 60

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR01/00549

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)
IPC7 C12N 15/70

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search trerms used)
NCBI pubmed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Leukemia. 1993 Feb;7(2):310-7	1-4, 5-6, 7-10
A	Cytotechnology. 1991 Sep;7(1):25-32.	1-4, 5-6, 7-10
A	J Immunol. 1992 Jul 1;149(1):113-9.	1-4, 5-6, 7-10
A	Int J Hematol. 1995 Feb;61(2):61-8.	1-4, 5-6, 7-10

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:
"A" *document defining the general state of the art which is not considered* to be of particular relevence
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" *document published prior to the international filing date but later* than the priority date claimed

"T" later document published after the international filing date or priority *date and not in conflict with the application but cited to understand* the principle or theory underlying the invention
"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 AUGUST 2001 (27.08.2001)	27 AUGUST 2001 (27.08.2001)

Name and mailing address of the ISA/KR	Authorized officer
Korean Intellectual Property Office Government Complex-Daejeon, Dunsan-dong, Seo-gu, Daejeon Metropolitan City 302-701, Republic of Korea Facsimile No. 82-42-472-7140	LIM, Hea Joon Telephone No. 82-42-481-5590

Form PCT/ISA/210 (second sheet) (July 1998)

PCT REQUEST

P0132-KAIST

Original (for SUBMISSION) - printed on 31.03.2001 10:35:39 AM

0	For receiving Office use only	
0-1	International Application No	
0-2	International Filing Date	
0-3	Name of receiving Office and 'PCT International Application'	
0-4	Form - PCT/RO/101 PCT Request	
0-4-1	Prepared using	PCT-EASY Version 2.91 (updated 01.01.2001)
0-5	Petition The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	Receiving Office (specified by the applicant)	Korean Industrial Property Office (RO/KR)
0-7	Applicant's or agent's file reference	P0132-KAIST
I	Title of invention	ESCHERICHIA COLI STRAIN SECRETING HUMAN GRANULOCYTE COLONY STIMULATING FACTOR(G-CSF)
II	Applicant	
II-1	This person is:	applicant only
II-2	Applicant for	all designated States except US
II-4	Name	KOREA ADVANCED INSTITUTE OF SCIENCE AND TECHNOLOGY
II-5	Address	373-1, Kusong-dong, Yusong-gu 305-701 Taejon Republic of Korea
II-6	State of nationality	KR
II-7	State of residence	KR
II-8	Telephone No.	82-42-869-5970
II-9	Facsimile No.	82-42-869-8800
III-1	Applicant and/or inventor	
III-1-1	This person is:	applicant and inventor
III-1-2	Applicant for	US only
III-1-4	Name (LAST, First)	LEE, Sang-Yup
III-1-5	Address	212-702 Expo Apartment Chonmin-dong, Yusong-gu 305-390 Taejon Republic of Korea
III-1-6	State of nationality	KR
III-1-7	State of residence	KR

PCT REQUEST

P0132-KAIST

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III-2	Applicant and/or inventor	
III-2-1	This person is	applicant and inventor
III-2-2	Applicant for	US only
III-2-4	Name (LAST, First)	JEONG, Ki-Jun
III-2-5	Address	102-411 KAIST Apartment Kung-dong, Yusong-gu 305-335 Taejon Republic of Korea
III-2-6	State of nationality	KR
III-2-7	State of residence	KR
IV-1	Agent or common representative; or address for correspondence The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as	agent
IV-1-1	Name (LAST, First)	LEE, Han-Young
IV-1-2	Address	8th Fl., Seowon Bldg. 1675-1 Seocho-dong, Seocho-gu 137-070 Seoul Republic of Korea
IV-1-3	Telephone No	82-2-596-7200
IV-1-4	Facsimile No	82-2-596-7280
IV-1-5	e-mail	LeePat@hitel.net
V	Designation of States	
V-1	Regional Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	EP: AT BE CH&LI CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE TR and any other State which is a Contracting State of the European Patent Convention and of the PCT
V-2	National Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	CN US
V-5	Precautionary Designation Statement In addition to the designations made under items V-1, V-2 and V-3, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except any designation(s) of the State(s) indicated under item V-6 below. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit.	
V-6	Exclusion(s) from precautionary designations	NONE

PCT REQUEST

P0132-KAIST

Original (for SUBMISSION) - printed on 31.03.2001 10:35:39 AM

VI-1	Priority claim of earlier national application	
VI-1-1	Filing date	31 March 2000 (31.03.2000)
VI-1-2	Number	2000-17052
VI-1-3	Country	KR
VI-2	Priority document request	
	The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s)	VI-1
VII-1	International Searching Authority Chosen	Korean Industrial Property Office (KIPO) (ISA/KR)

VIII	Check list	number of sheets	electronic file(s) attached
VIII-1	Request	4	-
VIII-2	Description (excluding sequence listing part)	16	-
VIII-3	Claims	2	-
VIII-4	Abstract	1	EZABST00.TXT
VIII-5	Drawings	13	-
VIII-6	Sequence listing part of description	13	-
VIII-7	TOTAL	49	
	Accompanying items	paper document(s) attached	electronic file(s) attached
VIII-8	Fee calculation sheet	✓	-
VIII-9	Separate signed power of attorney	✓	-
VIII-15	Nucleotide and/or amino acid sequence listing in computer readable form		separate diskette
VIII-16	PCT-EASY diskette	-	diskette
VIII-18	Figure of the drawings which should accompany the abstract	13	
VIII-19	Language of filing of the international application	Korean	
IX-1	Signature of applicant or agent		
IX-1-1	Name (LAST, First)	LEE, Han-Young	

FOR RECEIVING OFFICE USE ONLY

10-1	Date of actual receipt of the purported international application	JC18 Rec'd PCT/PTO 13 DEC 2001
10-2	Drawings:	
10-2-1	Received	
10-2-2	Not received	
10-3	Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application	
10-4	Date of timely receipt of the required corrections under PCT Article 11(2)	
10-5	International Searching Authority	ISA/KR

PCT REQUEST

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10-6	Transmittal of search copy delayed until search fee is paid	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	Date of receipt of the record copy by the International Bureau	

PCT (ANNEX - FEE CALCULATION SHEET)

P0132-KAIST

Original (for SUBMISSION) - printed on 31.03.2001 10:35:39 AM

(This sheet is not part of and does not count as a sheet of the international application)

0	For receiving Office use only	
0-1	International Application No	
0-2	Date stamp of the receiving Office	
0-4	Form - PCT/RO/101 (Annex) PCT Fee Calculation Sheet	
0-4-1	Prepared using	PCT-EASY Version 2.91 (updated 01.01.2001)
0-9	Applicant's or agent's file reference	P0132-KAIST
2	Applicant	KOREA ADVANCED INSTITUTE OF SCIENCE AND TECHNOLOGY, et al.

12	Calculation of prescribed fees		fee amount/multiplier	total amounts (KRW)	
12-1	Transmittal fee	T	⇨	45,000	
12-2	Search fee	S	⇨	150,000	
12-3	International fee Basic fee (first 30 sheets)	b1	425,800		
12-4	Remaining sheets		19		
12-5	Additional amount	(X)	9,800		
12-6	Total additional amount	b2	186,200		
12-7	b1 + b2 =	B	612,000		
12-8	Designation fees Number of designations contained in international application		3		
12-9	Number of designation fees payable (maximum 6)		3		
12-10	Amount of designation fee	(X)	91,700		
12-11	Total designation fees	D	275,100		
12-12	PCT-EASY fee reduction	R	-131,000		
12-13	Total international fee (B+D-R)	I	⇨	756,100	
12-14	Fee for priority document Number of priority documents requested		1		
12-15	Fee per document	(X)	0		
12-16	Total priority document fee	P	⇨	0	
12-17	TOTAL FEES PAYABLE (T+S+I+P)		⇨	951,100	
12-19	Mode of payment		cash		

VALIDATION LOG AND REMARKS

13-2-1	Validation messages Request	Green? A translation of the international application into English will have to be prepared under the responsibility of the ISA selected.

PCT (ANNEX - FEE CALCULATION SHEET) P0132-KAIST

Original (for SUBMISSION) - printed on 31.03.2001 10:35:39 AM

		Green? Please note that the entire request (including the title of invention) must be in English
13-2-2	Validation messages States	Green? More designations could be made. The following States have not been designated: AP:(GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW); EA:(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM); OA:(BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG); AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, LI, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW. Please verify.
13-2-6	Validation messages Contents	Green? The abstract shall be as concise as the disclosure permits (preferably 50 to 150 words if it is in English or when translated into English).
13-2-7	Validation messages Fees	Green? Please verify that modified fee amounts are correct.
13-2-10	Validation messages For receiving Office/International Bureau use only	Green? Verify electronic data for consistency against printed form.